RESEARCH OPEN ACCESS

Journal of Venomous Animals and Toxins including Tropical Diseases

ISSN 1678-9199

www.jvat.org

Immunomodulatory effect of *Tityus* sp. in mononuclear cells extracted from the blood of rheumatoid arthritis patients

Cindy Gabriela Rivera Tobar[1,3], Yisel del Mar Morales Urmendiz[1,3], Marcela Alejandra Vallejo[1], Diego Felipe Manquillo[1], Victoria Eugenia Niño Castaño[1], Ana Isabel Ospina Caicedo[2], Leydy Lorena Mendoza Tobar[3], Jimmy Alexander Guerrero Vargas[3,4], Rosa Amalia Dueñas Cuellar[1*]

[1]Grupo de Investigación en Inmunología y Enfermedades Infecciosas, Programa de Medicina, Departamento de Patología, Facultad de Ciencias de la Salud, Universidad del Cauca, Popayán, Colombia.

[2]Grupo de Investigación en Salud, Programa de Medicina, Departamento de Medicina Interna, Facultad de Ciencias de la Salud, Universidad del Cauca, Popayán, Colombia.

[3]Grupo de Investigaciones Herpetológicas y Toxinológicas (GIHT), Departamento de Biología, Facultad de Ciencias Naturales, Exactas y de la Educación, Universidad del Cauca, Popayán, Colombia.

[4]Grupo de Investigaciones Herpetológicas y Toxinológicas (GIHT), Centro de Investigaciones Biomédicas – Bioterio (CIBUC-Bioterio), Museo de Historia Natural, Departamento de Biología, Facultad de Ciencias Naturales, Exactas y de la Educación, Universidad del Cauca, Popayán, Colombia.

Keywords:
Rheumatoid arthritis
Venom
Tityus sp.
Tityus aff. *metuendus*
Immunomodulatory activity
T lymphocytes
Proliferation
Activation
Flow cytometry

ABSTRACT

Background: Pathophysiological mechanisms of rheumatoid arthritis arise because of a proinflammatory environment, generated by the interaction of autoreactive lymphocytes and proinflammatory mediators. Current strategies to mitigate the progression of the disease produce adverse effects, so there is a need for new therapeutic strategies and molecular targets to treat this disease. In this context, evidence suggests that scorpion venoms could modulate the immune response and some important cellular mechanisms of pharmacological interest. To evaluate the immunomodulatory effect of the venom of *Tityus* sp. (a possible new species close to *Tityus metuendus*) peripheral blood mononuclear cells of women diagnosed with RA were compared to cells of a control group.

Methods: A case-control study was conducted with samples of 10 women with a confirmed diagnosis of RA and controls matched by sex and age. The cytotoxicity of the venom was evaluated to find sublethal concentrations of the venom, and subsequently, their immunomodulatory capacity in terms of percentage of proliferation, cell activation, and cytokines production.

Results: the venom of *Tityus* sp. produced a decrease in the percentage of proliferation in the $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ cell subpopulations of RA patients and healthy controls, at concentrations of 252 and 126 µg/mL. However, the venom did not induce significant differences in the percentage of cell activation markers. The venom caused a decrease in IL-10 at a concentration of 252 µg/mL compared to untreated cells from patients and controls. The remaining cytokines did not show significant differences.

***Correspondence:** raduenasc@unicauca.edu.co

https://doi.org/10.1590/1678-9199-JVATITD-2023-0064

Received: 07 September 2023; Accepted: 10 June 2024; Published online: 16 September 2024

Conclusion: the venom of *Tityus* sp. is a potential source of molecules with immunomodulatory ability in CD4 and CD8 T lymphocytes. This result directs venom characterization studies to identify pharmacological targets with immunomodulatory capacity in T lymphocytes to enhance research in the treatment of autoimmune disorders such as RA.

Background

Rheumatoid arthritis (RA) is a global, chronic, inflammatory disease primarily affecting synovial membranes, leading to progressive damage to articular cartilage and bone. Autoreactive T and B cell activation triggers the release of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, and IL-17), crucial in RA's pathophysiology [1–4]. The condition, affecting approximately 1% of adults worldwide, is more common in the 40-60 age group, with occasional cases in juveniles [5]. Currently incurable, treatment involves symptom control (e.g., glucocorticoids) and disease-modifying drugs (DMARDs) to manage progression, despite associated adverse effects [6–14].

In RA's pathophysiology, the persistent activation of autoreactive T lymphocytes in the synovial membrane sustains the activation of other cells like synovial macrophages and fibroblasts, transforming them into destructive cells. Studies suggest a crucial role of effector and memory T lymphocytes (TEM) $CD4^{+}CD45RA^{-}CCR7^{-}$ in chronic inflammation, associated with proinflammatory cytokine production in the synovial membrane [15]. Simultaneously, potassium (K^{+}) voltage-dependent channels (Kv) are vital in T lymphocyte activation, helping sustained cell activation through membrane hyperpolarization and calcium (Ca^{+}) signaling, crucial for T cell differentiation [16, 17]. Significantly, Kv1.3 channel blockade has been linked to T lymphocyte inactivation and a subsequent reduction in effector cell function [16, 18].

Expression of Kv1.3 voltage-gated channels in T lymphocytes relies on differentiation state. In naive and central memory T cells, there are 400 to 500 Kv1.3 channels/cell, while effector and memory T cells (TEM) may express 1500 Kv1.3 channels/cell. This holds pharmacological interest given their role in autoimmune diseases and T cell activation [19]. The potassium channel KCa3.1, expressed at 10 channels/cell, also influences Ca+ signaling pathways in T cells. Differences in Kv1.3 channel numbers in TEM cells underscore their significance as potential therapeutic targets [17, 20]. As autoreactive TEMs contribute to autoimmune diseases, research focuses on finding Kv1.3 channel blockers as therapeutic targets for immunomodulating TEM cell responses in conditions like RA [17].

Potent potassium channel blockers, particularly peptides derived from scorpion venoms, have been extensively studied as potential immunomodulators. Casella Martins *et al.* [21], investigated the immunomodulatory effects of *Tityus serrulatus* venom at sub-lethal concentrations. Their findings revealed a decrease in T cell activation markers (CD69, CD25, and HLA-DR) and proliferation capacity, showing the immunomodulatory potential. Similar effects have been confirmed with various toxins from different scorpion species such as: a. Charibdotoxin, a peptide from the *Leiurus quinquestriatus hebraeus* venom [22, 23]; b. Iberiotoxin, a peptide from *Buthus tamulus* venom [24]; c. Kaliotoxin, a peptide from *Androctonus mauritanicus* venom [25]; d. Margatoxin peptide from *Centruroides margaritatus* venom [26]; e. OSK-1 (alpha-KTx3.7), a peptide from *Orthochirus scrobiculosus* venom [27]; and f. Vm24 (a-KTx 23.1), a peptide from *Vaejovis mexicanus smithi* venom [28]. All the above feature a Kv1.3 potassium channel blocking ability.

Considering this, Hashemlou *et al.* [29], conducted an *in vivo* study in Wistar rats in which arthritis was induced and treated with *Mesobuthus eupeus* venom, showing that there was a significant reduction in the arthritis index score in all treated animals. In particular, a decrease in the size of the tibiotarsal joint region was evidenced in the groups that received crude scorpion venom and the control group (Betamethasone). Similarly, Tanner *et al.* [30], determined that Iberiotoxin (IbTX) from the *Buthus tamulus* scorpion can significantly reduce the severity of RA in Wistar rats, based on histological observations of immune infiltrates, pannus extensions, hyperplasia, and erosion of rat cartilage, without inducing significant side effects.

Additionally, Tanner *et al.* [19], in a Wistar rat model of RA, showed that the blockade of the KCa1.1 channel, using Iberiotoxin (IbTX) reduces the ability of synovial fibroblasts to stimulate the proliferation and migration of T_{EM} cells. Likewise, the blockade of the Kv1.3 channel by ShK-186/Dalazatide reduces the ability of T_{EM} cells to produce mediators that act on synovial fibroblasts, promoting the decrease in the expression of KCa1.1 molecules and major histocompatibility complex (MHC) class II in these cells. Furthermore, combination therapy of potassium channel blockers targeting KCa1.1 and Kv1.3 is more effective than monotherapies in reducing disease severity in RA rat models.

Considering the compelling evidence highlighting the impact of scorpion venom components on immune response modulation in both *in vitro* and *in vivo* models [31–33], Colombia, with approximately 81 scorpion species in its biodiversity, holds particular interest. The endemic species *Tityus* sp., a possible new species close to *Tityus metuendus* found in the state of Cauca, lacks comprehensive data on its venom's chemical composition and biological activity, making it a valuable subject for research. Morales *et al.* [34], used a venom gland cDNA library to identify toxins in *Tityus* sp. venom, including potassium channel-specific toxins from subfamilies α-KTx15, α-12, and α-KTx18. Furthermore, four toxins from this venom demonstrated the ability to reduce K+ current in rat dorsal ganglion cells *in vitro*.

Therefore, *Tityus* sp. venom is a promising source of toxins of pharmacological interest. Their immunomodulatory ability with potential use in the treatment of autoimmune diseases, however, has yet to be explored. Considering the above, in the present study the immunomodulatory effect of venom of *Tityus* sp. (VTsp) was evaluated on the percentage of proliferation (in the $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ cell subpopulations) and activation percentage (in the $CD4CD69^+$, $CD4HLADR^+$, $CD8CD69^+$, and $CD8HLADR^+$) from peripheral blood mononuclear cells (PBMC) from female patients diagnosed with RA, compared with controls, as well as the presence of cellular mediators (IL-1β, IL-6, IL-10, and TNF-α) in culture supernatants.

Methods

Venom collection and preparation

50 adults of *Tityus* sp. scorpions (male and female) were collected in the city of Popayán. The specimens were taken to the *Centro de Investigaciones Biomédicas* – vivarium (CIBUC) of the Universidad del Cauca where they were provided with food and water *ad libitum*. The VTsp was extracted using electrostimulation (Lafayette instruments) [35] at 30 V direct current, lyophilized (FreeZone 2.5 - LABCONCO, USA), and stored at -70 °C until its use.

The lyophilized venom was diluted with 200 µL of 1X phosphate-buffered saline (PBS) (Gibco/Invitrogen, Van Allen Way, Carlsbad, CA, USA) then homogenized (Heidolph, USA) and subsequently transferred to a sterile 15 mL tube (Falcon BD, Franklin Lakes, NJ, USA). Quantification of the complete venom was performed by reading at 280/260 nm in a microplate spectrophotometer (Multiskan SkyHigh Photometer - Thermo Fisher Scientific, USA).

Isolation and culture of peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from blood taken by venipuncture, by the discontinuous density centrifugation method via Ficoll-Hypaque (Hystopaque®-1077; Sigma Aldrich, USA). The PBMCs were washed twice and resuspended in RPMI 1640 medium (Gibco, USA). 150,000 cells/200 µL were cultured per well in a 96-well microplate (Fisher Scientific, USA) in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% Penicillin/Streptomycin (BioWhittaker, USA), and stimulated with Phytohemagglutinin (PHA; 2.5 µg/mL; Sigma-Aldrich, USA) in the presence or otherwise of different concentrations of *Tityus* sp. venom at 37 °C and 5% CO_2, for 72 hours.

Evaluation of the cytotoxic activity of *Tityus* sp. venom on PBMC and determination of inhibitory concentration 50 (IC_{50})

The VTsp cytotoxicity test was performed using the resazurin method [36]. PBMC from healthy donors, under culture conditions described above, were exposed to different concentrations of the *Tityus* sp. venom (2000; 1000; 500; 250; 125; 62.5; 31.2; 15.6; 7.8 and 3.9 µg/mL) for 72 hours at 37 °C and 5% CO_2. Untreated cells were used as the negative control and Triton 1X (Sigma-Aldrich, USA) as a positive control. 16 hours before the end of the culture time, resazurin (Alamar-Blue; Invitrogen, USA) was added at 10% v/v in each well. After 72 hours of culture, readings were done at 570 nm and 630 nm in a microplate spectrophotometer (Multiskan SkyHigh Photometer - Thermo Fisher Scientific, USA). The percentage reduction in cell viability was calculated based on the oxidation/reduction formula [37]. The effect of the venom was expressed in percentages of cell viability concerning the negative control.

Determination of the IC_{50} was established as the concentration in which cell viability was reduced by 50%. It was calculated using GraphPad Prism 8 software, taking as variables the concentration of venom, mortality, and control groups. From the IC_{50} value, three sublethal concentrations were taken that corresponded to 30, 15, and 7.5% of the IC_{50} value, respectively; considering a percentage of viability greater than 80% for PBMC, such that cell integrity could be ensured in the 72 hours of culture.

Recruitment of patients with RA and social and clinical profile analysis

Ten females diagnosed with RA (18 to 69 years old) from the State of Cauca, were recruited from the private consultation of rheumatologist Ana Isabel Ospina, as defined in the criteria for the diagnosis of rheumatoid arthritis provided by the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) in 2010 [38, 39]. Additionally, ten healthy females were recruited as control and matched by age and sex with the patients. The exclusion criteria included not presenting symptoms referring to any acute or chronic disease, and not having consumed any non-steroidal anti-inflammatory drugs (NSAIDs) and/or disease-modifying drugs (DMARDs) for at least four months. Both patients and controls were interviewed using a sociodemographic survey. All study participants signed informed consent, in accordance with the ethics endorsement issued by the Universidad del Cauca (ID-4783).

Immunophenotyping of lymphocyte subpopulations and activation markers

Immunophenotyping was performed using flow cytometric analysis to identify $TCD4^+$ and $TCD8^+$ lymphocyte subpopulations and measure levels of activation markers in PBMC from RA patients as controls. For this, the cells ($5x10^6$ cells per well) that were seeded in the presence or otherwise of VTsp (252; 126; 63 µg/mL) for 72 hours at 37 °C and with 5% CO_2, were collected and washed with 200 µL of buffer (1X PBS; Gibco/Invitrogen, Van Allen Way, Carlsbad, CA, USA) and labeled with anti-CD4-APC and anti-CD8-PECy7; and anti-CD69-FITC and anti-HLA-DR-PE antibodies; (BD Bioscience, USA). The cells were then incubated for 30 minutes in the dark. Two washes were performed and resuspended in 1x PBS.

For the flow cytometry analysis, a total of 20,000 events were acquired in the flow cytometer (Accuri C6 flow cytometer - BD Biosciences, USA). The analysis strategy was carried out as follows: the gate for the lymphocytes was selected according to the forward and side scatter distribution and the percentage of $CD4^+CD69^+$, $CD4^+$ $HLA\text{-}DR^+$, $CD8^+CD69^+$, and $CD8^+$ $HLA\text{-}DR^+$ cells were identified using a Dotplot graph.

Evaluation of cell proliferation

For the cell proliferation test, the staining protocol with 5(6)-Succinimidyl Ester Diacetate of Carboxyfluorescein/CFSE (Molecular Probes/Invitrogen, Van Allen Way, Carlsbad, CA, USA), modified from Lyons [40], was performed. About $5x10^6$ cells/mL were collected in 15 mL tubes. They were then washed and resuspended in 1X PBS to perform the labeling with CFSE (0.062 µM; Molecular Probes, USA) for 15 minutes at 37 °C. Two washes were then performed; the first with 1X PBS and the second with complete RPMI 1640 medium. The cells were then resuspended in RPMI 1640 complete medium and incubated for 30 minutes at 37 °C.

The PBMCs previously labeled with CFSE were cultured in a 96-well microplate (Fisher Scientific, USA) in RPMI 1640 complete medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% Penicillin/Streptomycin (BioWhittaker, USA), and stimulated with Phytohemagglutinin (PHA; 2.5 µg/mL; Sigma-Aldrich, USA) in the absence or otherwise of *Tityus* sp. venom (252, 126 and 63 µg/mL) at 37 °C, under 5% CO_2, for 72 hours. Then, the cells were labeled with anti-CD3-PE, anti-CD4-APC, and anti-CD8-PECy7 monoclonal antibodies (BioLegend, USA) and incubated for 30 minutes in the dark. Reading by flow cytometry (Accuri C6 flow cytometer - BD Biosciences, USA).

The analysis strategy to identify the percentage of cells in proliferation was carried out as follows: the region of the lymphocytes was identified through a gate, considering forward and side scatter, and using a Dotplot graph and histogram, the percentage was quantified of $CFSE^{low}$ lymphocytes in the $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ subpopulations.

Cytokine quantification

The cytokines IL-6, TNF-α, IL1-β, and IL-10 were quantified by enzyme-linked immunosorbent assay (ELISA) using ELISA MaxTM Deluxe Set kits (BioLegend®, San Diego, CA 92121, USA) from PBMC culture supernatants (72 hours; 37 °C; 5% CO_2), treated with PHA (2.5 µg/mL) in the presence or otherwise of VTsp (252; 126; 63 µg/mL). The procedure was conducted according to manufacturer specifications and the reading was carried out in the EPOCH 2 spectrophotometer (Agilent Biotek, USA).

Statistical analysis

The results were analyzed using a two-way analysis of variance (ANOVA), considering a significance value of $p < 0.05$, followed by the Tukey multiple comparison test. Bar graphs were prepared in which the mean and the standard error of the mean (± SEM) were expressed for each condition and the sociodemographic analysis of the patients with RA and healthy controls were analyzed using bivariate analysis in GraphPad Prism 8 statistical analysis software.

Results

Determination of inhibitory concentration 50 and sublethal concentrations of *Tityus* sp. venom

The percent cell viability in the presence of the different concentrations of venom was reduced on a dose basis depending on the concentrations of VTsp used compared to the control (untreated cells), with 2000 and 1000 µg/mL being the concentrations with the greatest percent drop in viability. The IC_{50} calculation yielded a concentration equivalent to 840.3 µg/mL (Figure 1). Considering the above, concentrations 252, 126, and 63 µg/mL were selected; corresponding to 30, 15, and 7.5% of the IC_{50} value, as sublethal concentrations to conduct the study of immunological variables.

Social and clinical profile analysis of AR patients and controls

In the sociodemographic analysis, it was found that the ten patients with RA (n = 10: 100%) had an average age of 41 ± 0.51 years, and the age range was 20-69 years. The most recurrent occupations among RA patients were student (n = 2: 20%), housewife (n = 3: 30%), independent (n = 2: 20%), and professionals (n = 3 30%). Additionally, the clinical findings show that RA patients suffer pain and/or swelling in large joints

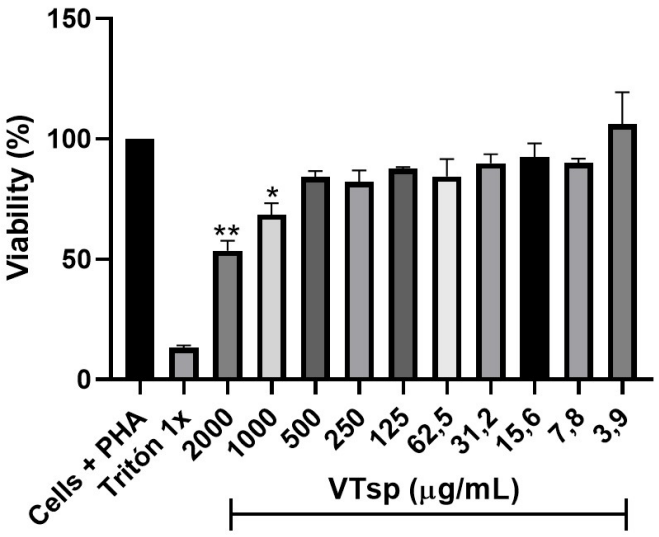

Figure 1. Effect of *Tityus* sp. venom (VTsp) on the cell viability of peripheral blood mononuclear cells (PBMC), that were stimulated with PHA (Phytohemagglutinin), and exposed to the VTsp in a 72h culture, using the resazurin fluorimetric assay (AlamarBlue). Negative control: Triton 1x; positive control: untreated cells. Results were expressed as the standard error of the mean (± SEM). Significance values $p < 0.05$* and $p < 0.01$**.

where 90% (n = 9) reported in the knee, followed by shoulders (n = 8: 80%), ankles (n = 8: 80%), hips, and elbows (n = 7: 70%) and in small joints, where 100% (n = 10) of the patients had problems in the metacarpophalangeal joints, as well as wrists (n = 9: 90%), proximal interphalangeal joints (n = 8: 80%) and metatarsophalangeal joints (n = 3: 30%).

The *Tityus* sp. venom did not induce changes in the percentage of PBMC activation markers

The subpopulations of $CD4^+$ and $CD8^+$ T cells from patients with RA and healthy controls, stimulated with PHA and treated or otherwise with *Tityus* sp. venom, did not show significant changes in the percentage of expression of the activation markers $CD69^+$ and $HLADR^+$. Comparing the activation markers, regardless of treatment, it was seen that the percentage of $CD8^+$ $HLADR^+$ cells was lower in RA patients compared to controls, with a statistical difference of $p < 0.05$ (Figure 2).

Tityus sp. venom induced a decrease in the percentage of proliferation of the subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes

On evaluating the immunomodulatory capacity of the three concentrations of *Tityus* sp. venom regarding cell proliferation, the statistical analysis showed a significant difference ($p < 0.05$). The multiple comparison analysis showed a significant decrease between proliferation percentages for $CFSE^{low}$ cell subpopulations in $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ lymphocytes, in the untreated condition compared to cells treated with the concentrations 252 µM/mL and 126 µM/mL of the venom ($p < 0.01$) (Figures 3 and 4).

Tityus sp. venom induced a decrease in the release of cytokine IL-10

On analyzing the expression of the cytokines IL-6, TNF-α, IL1-β, and IL-10 (Figure 5), a significant decrease in the level of

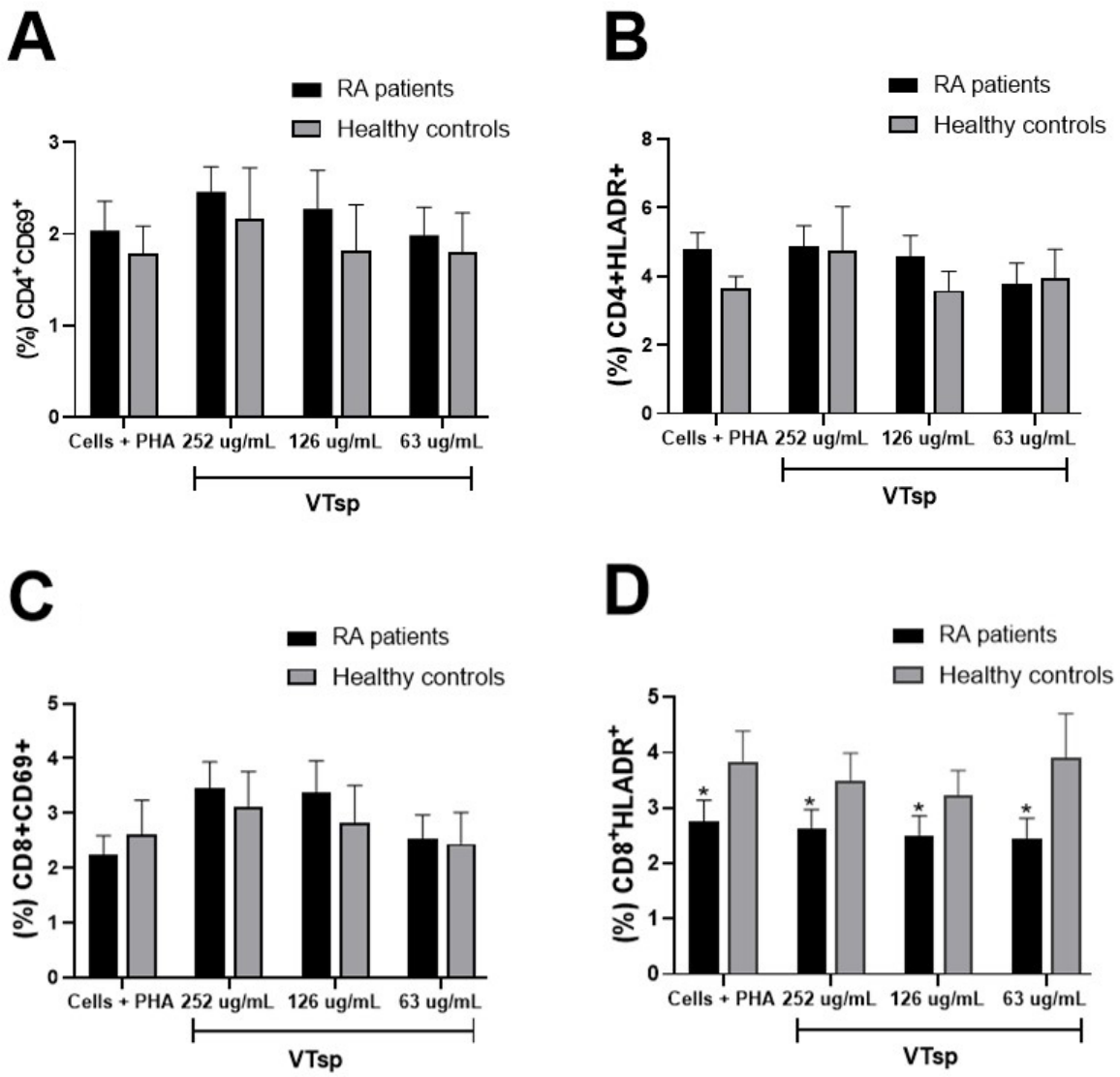

Figure 2. Percentage of cell activation of peripheral blood mononuclear cells (PBMC) that were stimulated with PHA (Phytohemagglutinin), and exposed to the VTsp, in a 72h culture. Activation analysis of **(A)** $CD4^+CD69^+$, **(B)** $CD4^+HLADR^+$, **(C)** $CD8CD69^+$, **(D)** $CD8^+HLADR^+$, cell populations exposed to *Tityus* sp. venom (252, 126, and 63 µg/mL) comparing RA patients and healthy controls.

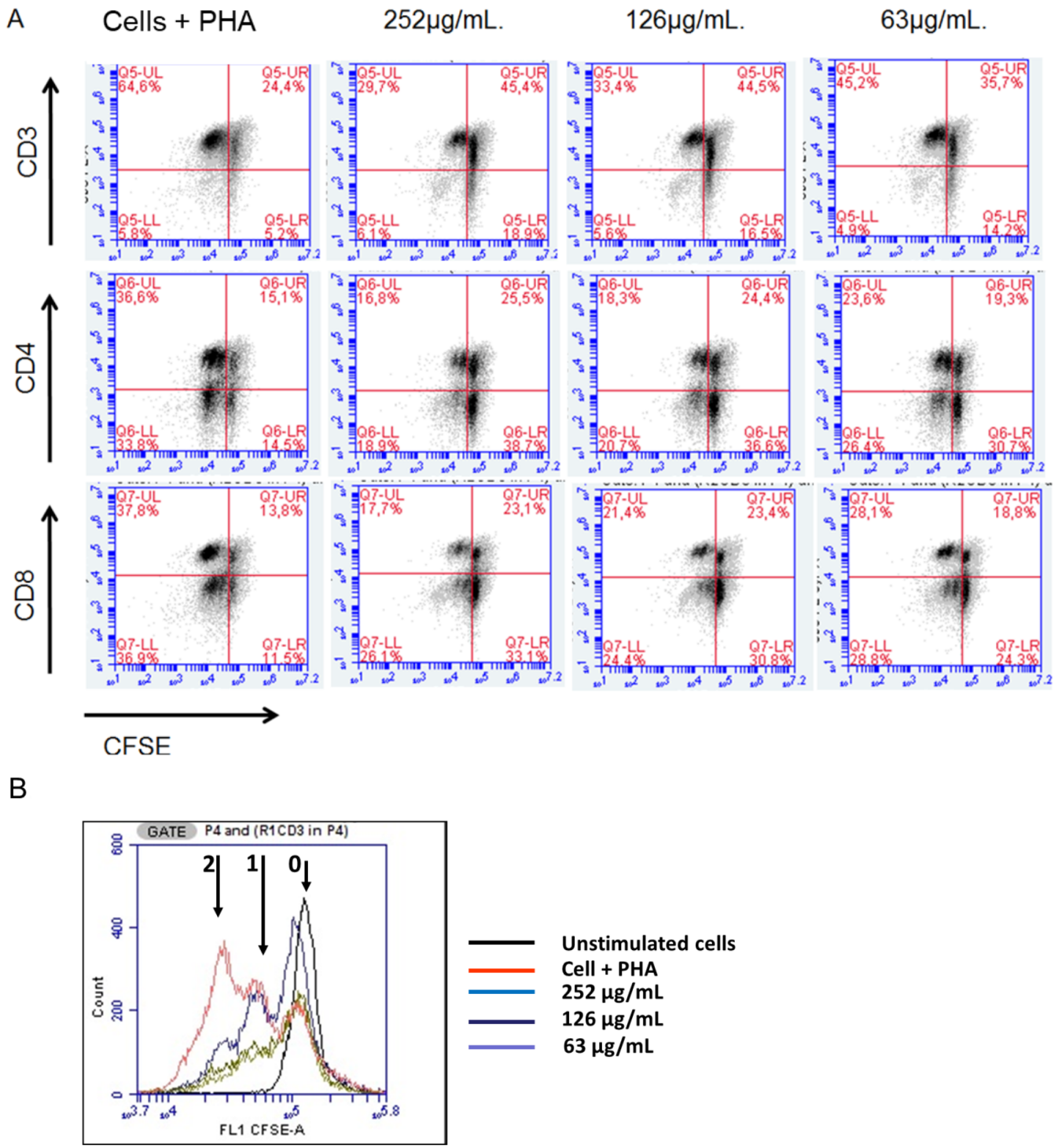

Figure 3. Effect of *Tityus* sp. venom on the percentage of PBMC proliferation; **(A)** Dotplot representative of the cytometric analysis to assess the percentage of cell proliferation in the $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ populations that were stimulated with PHA (Phytohemagglutinin), marked with CFSE, and exposed to *Tityus* sp. venom in a 72-hour culture; **(B)** Representative histogram of the effect of *Tityus* venom on PBMC proliferation. The arrows refer to the number of cell cycles that occurred during the 72 hours.

cytokine IL-10 was observed in the cells treated with 252 µg/mL of the venom compared to untreated cells in RA patients and healthy controls (Figure 5D). No significant differences were observed in the concentration of IL-6, TNF-α, and IL1-β, in any of the conditions evaluated. In the comparison of the levels of cytokines between patients and controls and independently of the experimental conditions, a significant increase in the level of the cytokine IL-6 was found in RA patients compared to healthy controls ($p < 0.05$) (Figure 5A).

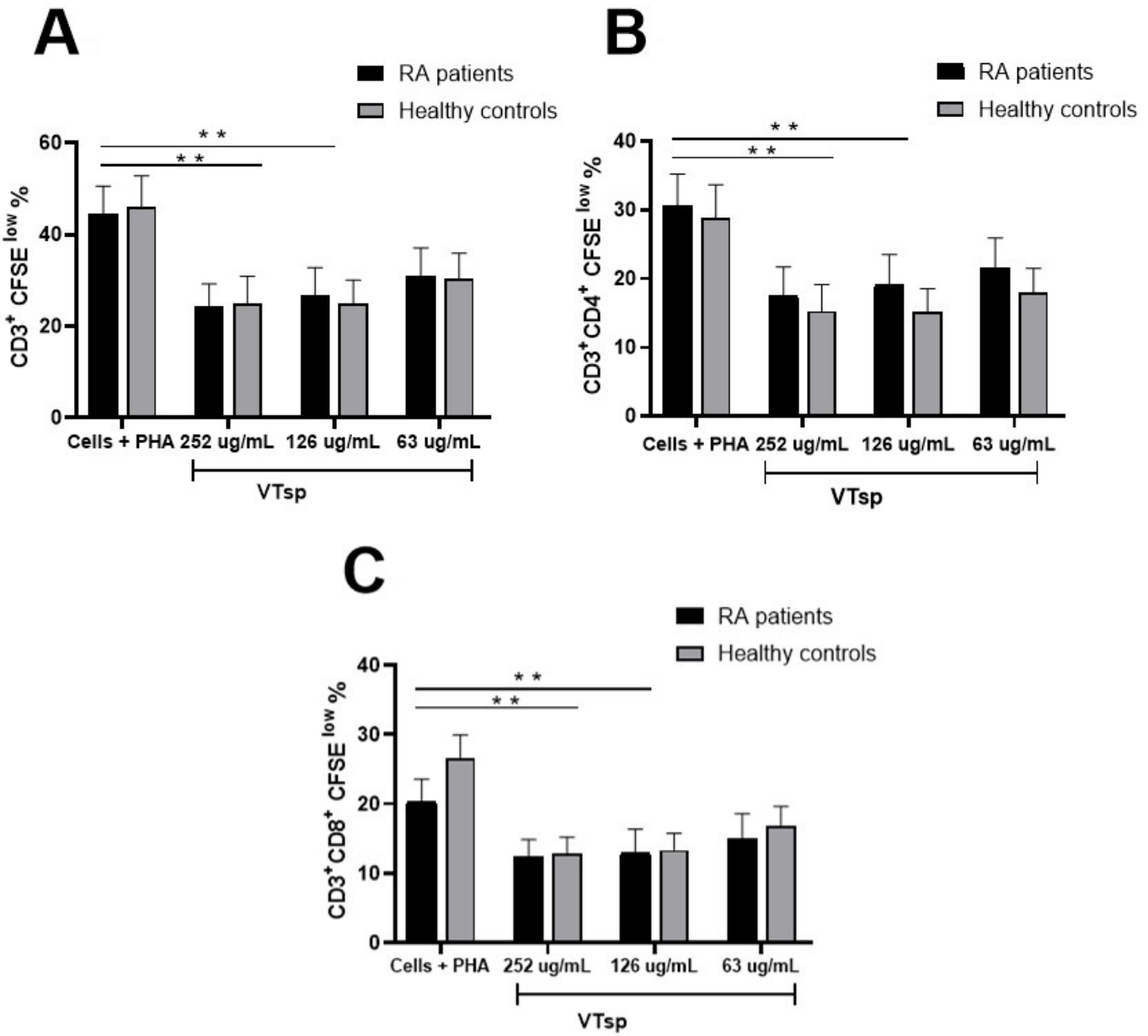

Figure 4. Percentage of the proliferation of PBMC treated with *Tityus* sp. venom. Proliferation analysis of **(A)** $CD3^+$, **(B)** $CD3^+CD4^+$, **(C)** $CD3CD8^+$ $CFSE^{low}$ cell populations, which were stimulated with PHA (Phytohemagglutinin) and exposed to *Tityus* sp. venom (252, 126 and 63 µg/mL) comparing RA patients and healthy controls. Results are expressed as mean standard error (± MSE). Significance value $p < 0.01$**.

DISCUSSION

The present study evaluated the immunomodulatory activity of the venom of the *Tityus* sp. scorpion on the ability for cell proliferation and activation in populations of $CD8^+$ and $CD4^+$ T lymphocytes, from patients diagnosed with RA and controls, as well as the production of cytokines IL-6, TNF-α, IL1-β, IL-10, in 72h cell culture supernatants. The results obtained show that the venom-induced significant changes and responses regarding cell proliferation and cytokine secretion independently of the state of disease.

Determination of the IC_{50} of *Tityus* sp. venom *in vitro* assays was crucial to assess its toxicity and conduct more controlled research processes, mainly considering that, in the case of this research, it was necessary to start from cells with viability greater than 80% to measure the functional conditions of immune response cells. To our knowledge, to date, this is the first report of IC_{50} for *Tityus* sp. venom *in vitro* assays.

The results of the present study did not show differences in the percentage of cells that express CD69 and HLA-DR between the different treatments carried out on PBMC of RA patients

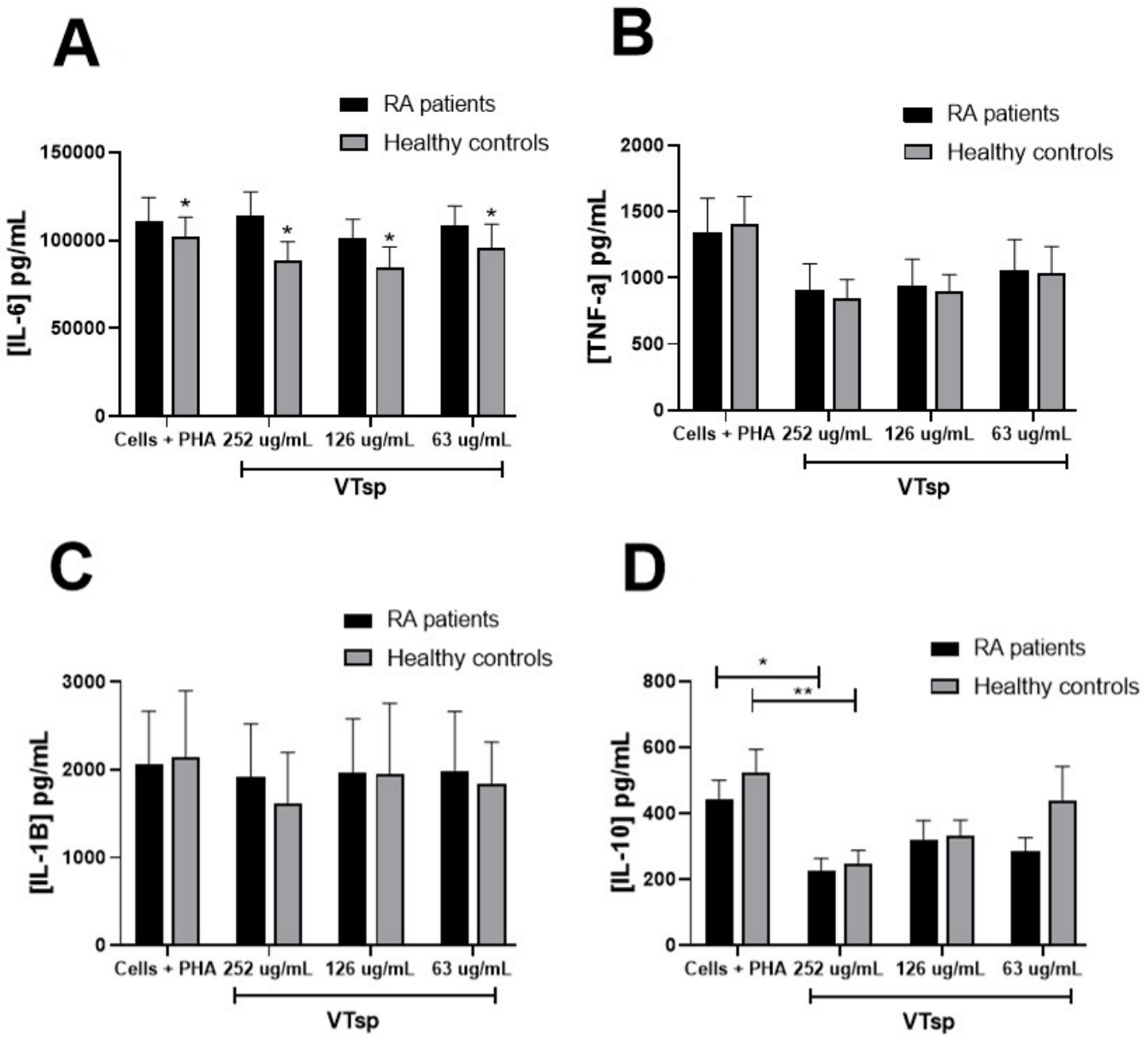

Figure 5. Analysis of cytokine expression of PBMC from RA patients and healthy controls exposed to VTsp venom. Production of cytokines **(A)** IL-6, **(B)** TNF-α, **(C)** IL1-β, and **(D)** IL-10 expressed in pg/mL of mononuclear cells from patients diagnosed with RA and healthy patients cultured with PHA and treated with *Tityus* sp. Results are expressed as the mean standard error (± MSE). Significance values were $p < 0.01$** and $p < 0.05$*.

when compared with the treatments carried out on the PBMC of healthy controls. However, when the percentage of $CD8^+$ $HLADR^+$ cells was compared between the groups analyzed, it was found that in RA patients the percentage was lower compared to controls, indicating that the lower percentage of $CD8^+HLADR^+$ cells is associated with the disease and not with the treatment with the different concentrations of the venom. These can be explained by studies that show correlations between the severity of RA and the number and phenotype of $CD8^+$ T cells in peripheral blood, or in inflammatory tissue [41]. Other research mentions that, in patients with early RA, the absolute number of $CD8^+$ T cells is higher than in healthy controls; while in patients in remission, the number of $CD8^+$ T lymphocytes is reduced [42].

We can recall that VTsp venom is composed of, among other molecules, specific toxins for potassium channels classified in the α-KTx15, α-12, and α-KTx18 subfamilies, capable of decreasing the K+ current in rat dorsal ganglion cells *in-vitro* [34], As such, about the proliferation results, the significant decrease between proliferation percentages for $CFSE^{low}$ cell subpopulations in $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes in untreated condition compared with cells treated with venom concentrations of 252 µg/mL and 126 µg/mL may be related to the fact that certain components of the venom may be highly

selective for Kv1.3 channels. This relates to the modulation of the cellular response of the T lymphocytes [43] since it has been shown that the selective blockade of K+ channels (mainly Kv1.3) leads to depolarization of the membrane and inhibits the influx of Ca^{2+}, resulting in a diminution of cell proliferation and of its effector functions [19, 44].

Considering this, the study by Varga *et al.* [45] demonstrated that the Vm24 toxin, from the scorpion *Vaejovis mexicanus smithi*, inhibited the proliferation of T lymphocytes stimulated with anti-CD3/CD28, after 96h of culture in all the concentrations evaluated (100, 50, 10, 5, 1 and 0.1 pM; likewise, the toxin inhibited the expression of the CD25 marker in stimulated T $CD4^+$ lymphocytes. In addition, the authors verified that Vm24 can inhibit the Kv1.3 channels of human lymphocytes with high affinity, with a selectivity 1500 times greater than other channels such as KCa3.1, Kv1.1, and Kv1.2; as well as reducing delayed-type hypersensitivity (DTH) reactions in *in-vivo* models.

Additionally, it is noteworthy that since Kv.1.3 is a predominant potassium channel in resting T cells (naïve or T_{EM}), proliferation is sensitive to inhibition of this channel, however, it is not complete, which may be an indicator of the presence of other channels in cells [45–47].

The obtained results also correlate with those obtained by Casella Martins *et al.* [21], who reported a decrease in the percentage of proliferation in populations of PBMC $CD4^+CD25^+$ T lymphocytes treated with *Tityus serrulatus* venom (concentrations of 50 and 100 µg/mL), compared to the control (cells + PHA). Ion channels are known to be involved in the early phase of lymphocyte activation by Phytohemagglutinin (PHA). Since PHA is a mitogen that acts by activating T lymphocytes by binding to the TCR receptor, it is possible that the toxins present in *Tityus serrulatus* venom blocked the K+ channels of $CD4^+CD25^+$ cells and impaired PHA action and consequently the activation percentage, as in this study [16, 21].

The *Tityus* sp. venom, as demonstrated in the chromatographic profiles obtained by Duque-Morales [34], and Arenas [48], has a profile that follows the pattern reported by Barona *et al.* [49], for *Tityus pachyurus* and Guerrero Vargas *et al.* [50] for *Tityus obscurus*, showing the region from 10 to 30 min (retention time) with fractions corresponding to toxins that act on potassium channels, therefore, it is possible to infer that the venom of *Tityus* sp. may have one or more toxins that act on Kv1.3 potassium channels. Based on this inference, and with the results obtained in this study, the venom of *Tityus* sp present a pharmacological and biotechnological potential as demonstrated in other closed species of scorpions [51].

Specifically, Kv1.3 potassium channels are considered a new therapeutic target to treat and diagnose inflammatory diseases, given their crucial role in T lymphocytes [52]. Based on the results obtained in this work, the venom of *Tityus* sp. and its toxins with action on Kv1.3 could be promising immunomodulatory molecules that can be used in treatments for autoimmune diseases, an aspect consistent with Zhao *et al.* [53] and Harunur *et al.* [54].

Investigations have shown that some scorpion toxins and complete venoms can inhibit certain proinflammatory cytokines and/or enhance the production of anti-inflammatory cytokines such as the IL-10 cytokine, both in *vivo* and *ex vivo* [32, 55, 56]. However, contrary to these findings, in the present study a decrease in the concentration of IL-10 was obtained in PBMC culture supernatants exposed to a concentration of 252 µg/mL of *Tityus* sp. venom, both in patients with RA and healthy patients. This result matches with the study of Veytia-Buchelli *et al.* [57], who treated T_{EM} $CD4^+$ lymphocytes isolated from healthy donors with the Vm24 toxin from the *Vaejovis mexicanus* scorpion and found a decrease in the production of this cytokine, consistent with an inhibition of the transcription factor NFATc2 (Nuclear factor of activated T cells) and the recruitment of IRF4 (Interferon regulatory factor 4); two transcription factors involved in T cell activation, proliferation, and differentiation, which synergistically increase the activity of the Th2-specific enhancer, CNS-9 (a regulatory element downstream of the IL-10 gene locus).

Finally, the increase in IL-6 in patients with RA compared to healthy controls coincides with the results obtained by Wang *et al.* [58], who report an increase in IL-6 levels obtained from PBMC culture supernatants of 24h, stimulated with PHA in patients with RA compared to healthy controls. Usually, this cytokine is associated with inflammatory processes and is found to be in abundance in the synovial fluid and the serum of patients with RA. The increase in its levels has been correlated with the activity and progression of the disease and joint destruction [59], therefore, their increased presence in culture supernatants from people with RA is synonymous with the inflammatory processes characteristic of the disease.

CONCLUSIONS

The results of this study showed a strong suggestion of an immunomodulatory effect induced by *Tityus* sp. venom on PBMCs, both in patients with RA and in healthy patients, mainly in cell proliferation (concentrations of 252 and 126 µg/mL) and in the decrease of cytokines such as IL-10, possibly associated with the presence of toxins that act on ionic channels, mainly potassium K+. IL-6 cytokine levels were higher in RA patients compared to healthy controls, confirming the inflammatory state associated with the disease.

Considering that this study shows the immunomodulatory potential of the venom of the *Tityus* sp. scorpion, the need to carry out detailed investigations of its components is highlighted to identify which ones participate in the process of modulating the response of T lymphocytes, that, in the context of rheumatoid arthritis, as well as in other autoimmune diseases, has a major role in pathophysiology. As such, the study of new therapeutic alternatives for RA is a vital field in which the prospective research of the venom of this species acquires relevance as a possible therapeutic agent.

Acknowledgments

The authors thank the Centro de Investigaciones Biomédicas of the University of Cauca for the support in the use of the facilities for the maintenance of scorpions and the Herpetological and Toxicological Research Group for accompanying us in the process of collecting specimens, care, and in the developing processes of the scorpions, as well as in our training.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding

The present research was supported by the Research Vice-Rectorate of the University of Cauca, identified with ID 4783, under the "Cultivating Seeds" funding call.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CGRT made substantial contributions to the acquisition, analysis, and interpretation of data for the work; and wrote the initial version of the article. YMMU made substantial contributions to the acquisition, analysis, and interpretation of data for the work; and wrote the initial version of the article. VENC wrote the fundraising project; carried out a critical review of the intellectual content and made substantial contributions to the analysis and interpretation of data. MAV and DFM made substantial contributions to the acquisition analysis and interpretation of data related to the clinical conditions of the patients. AIOC and JAGV approved the definitive version of the manuscript. LLMT wrote the fundraising project and approved the definitive version of the manuscript. RADC wrote the fundraising project; performed a critical review of the intellectual content and made substantial contributions to the analysis and interpretation of data.

Ethics approval and consent to participate

The present study was approved by the University of Cauca, under project ID-4783, and is covered by the animal collection permit of the University of Cauca, covered by the Framework Permit for the collection of specimens of Biological diversity for non-commercial scientific research purposes, the National Authority for Environmental Licenses protected by decree 1076 of 2015 article 2.2.2.8.2.6.

Consent for publication

The patients participating in the research signed informed consent for the management of the data resulting from the research.

References

1. McInnes IB, Schett G. Mechanism of Disease The Pathogenesis of Rheumatoid Arthritis. N Engl J Med. 2011 Dec 8;365(23):2205–19.
2. Scott IC, Machin A, Mallen CD, Hider SL. The extra-articular impacts of rheumatoid arthritis: moving towards holistic care. BMC Rheumatol. 2018 Oct 30;2(1):32.
3. Alunno A, Carubbi F, Giacomelli R, Gerli R. Cytokines in the pathogenesis of rheumatoid arthritis: new players and therapeutic targets. BMC Rheumatol. 2017;1(1):1–13.
4. Croia C, Bursi R, Sutera D, Petrelli F, Alunno A, Puxeddu I. One year in review 2019: pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol. 2019 May-Jun;37(3):347-57.
5. Kvien TK, Uhlig T, Ødegård S, Heiberg MS. Epidemiological Aspects of Rheumatoid Arthritis: The Sex Ratio. Ann N Y Acad Sci. 2006 Jun;1069(1):212–22.
6. Smolen JS, Landewé R, Bijlsma J, Burmester G, Chatzidionysiou K, Dougados M, Nam J, Ramiro S, Voshaar M, van Vollenhoven R, Aletaha D, Aringer M, Boers M, Buckley C, Buttgereit F, Bykerk V, Cardiel M, Combe B, Cutolo M, van Eijk-Hustings Y, Emery P, Finckh A, Gabay C, Gomez-Reino J, Gossec L, Gottenberg JE, Hazes JM, Huizinga T, Jani M, Karateev D, Kouloumas M, Kvien T, Li Z, Mariette X, McInnes I, Mysler E, Nash P, Pavelka K, Poór G, Richez C, van Riel P, Rubbert-Roth A, Saag K, da Silva J, Stamm T, Takeuchi T, Westhovens R, de Wit M, van der Heijde D. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update Josef. Ann Rheum Dis. 2013;73(6):492–509.
7. Aletaha D, Alasti F, Smolen JS. Optimisation of a treat-To-Target approach in rheumatoid arthritis: Strategies for the 3-month time point. Ann Rheum Dis. 2016 Aug;75(8):1479–85.
8. Lopez-Olivo MA, Tayar JH, Martinez-Lopez JA, Pollono EN, Cueto JP, Gonzales-Crespo MR, Fulton S, Suarez-Almazor ME Risk of malignancies in patients with rheumatoid arthritis treated with biologic therapy: A meta-analysis. JAMA. 2012 Sep 5;308(9):898-908.
9. Kremer JM, Romain PL. Major side effects of low-dose methotrexate. UpToDate, Post TW (Ed). 2020. Available from: https://www.uptodate.com/contents/major-side-effects-of-low-dose-methotrexate#.
10. Fox R, Helfgott SM. Pharmacology, dosing, and adverse effects of leflunomide in the treatment of rheumatoid arthritis. UpToDate. Post TW (Ed). 2019.
11. Linares V, Alonso V, Domingo JL. Oxidative stress as a mechanism underlying sulfasalazine-induced toxicity. Expert Opin Drug Saf. 2011 Mar;10(2):253–63.
12. Fleischmann R, van Vollenhoven RF, Vencovský J, Alten R, Davies O, Mountian I, de Longueville M, Carter D, Choy E. Long-Term Maintenance of Certolizumab Pegol Safety and Efficacy, in Combination with Methotrexate and as Monotherapy, in Rheumatoid Arthritis Patients. Rheumatol Ther. 2017 Jun;4(1):57–69.
13. Keystone EC, Breedveld FC, Kupper H, Li Y, Florentinus S, Sainsbury I. Long-term use of adalimumab as monotherapy after attainment of low disease activity with adalimumab plus methotrexate in patients with rheumatoid arthritis. RMD Open. 2018 Jun 13;4(1):e000637.
14. Schaible TF. Long term safety of infliximab. Can J Gastroenterol. 2000 Sep;14 Suppl C:29C-32C.
15. Fasth AER, Cao D, Van Vollenhoven R, Trollmo C, Malmström V. CD28nullCD4+ T cells--characterization of an effector memory T-cell population in patients with rheumatoid arthritis. Scand J Immunol. 2004;60(1–2):199–208.
16. Chandy KG, Cahalan M, Pennington M, Norton RS, Wulff H, Gutman GA. Potassium channels in T lymphocytes: toxins to therapeutic immunosuppressants. Toxicon. 2001 Sep;39(9):1269–76.
17. Panyi G, Varga Z, Gáspár R. Ion channels and lymphocyte activation. Immunol Lett. 2004 Mar 29;92(1–2):55–66.
18. Ghanshani S, Wulff H, Miller MJ, Rohm H, Neben A, Gutman GA, Cahalan MD, Chandy KG. Up-regulation of the IKCa1 Potassium Channel during T-cell Activation. J Biol Chem. 2000 Nov 24;275(47):37137–49.
19. Oliveira IS, Ferreira IG, Alexandre-Silva GM, Cerni FA, Cremonez CM, Arantes EC, Zottich U, Pucca MB. Scorpion toxins targeting Kv1.3 channels:

Insights into immunosuppression. J Venom Anim Toxins incl Trop Dis. 2019;25:e148118. doi: 10.1590/1678-9199-JVATITD-1481-18.

20. Feske S, Skolnik EY, Prakriya M. Ion channels and transporters in lymphocyte function and immunity. Nat Rev Immunol. 2012;12(7):532–47.
21. Casella-Martins A, Ayres LR, Burin SM, Morais FR, Pereira JC, Faccioli LH, Sampaio SV, Arantes EC, Castro FA, Pereira-Crott LS. Immunomodulatory activity of *Tityus serrulatus* scorpion venom on human T lymphocytes. J Venom Anim Toxins incl Trop Dis. 2015 Nov 11;21(1):46. doi: 10.1186/s40409-015-0046-3.
22. Sands SB, Lewis RS, Cahalan MD. Charybdotoxin blocks voltage-gated K+ channels in human and murine T lymphocytes. J Gen Physio. 1989 Jun 1;93(6):1061–74.
23. Price M, Lee SC, Deutsch C. Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes. Proc Natl Acad Sci U S A. 1989 Dec 1;86(24):10171–5.
24. Giangiacomo KM, Garcia ML, McManus OB. Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. Biochemistry. 1992 Jul;31(29):6719–27.
25. Valverde P, Kawai T, Taubman MA. Selective blockade of voltage-gated potassium channels reduces inflammatory bone resorption in experimental periodontal disease. J Bone Miner Res. 2004 Jan;19(1):155–64.
26. Garcia-Calvo M, Leonard RJ, Novick J, Stevens SP, Schmalhofer W, Kaczorowski GJ, Garcia ML. Purification, characterization, and biosynthesis of margatoxin, a component of *Centruroides margaritatus* venom that selectively inhibits voltage-dependent potassium channels. J Biol Chem. 1993 Sep 5;268(25):18866–74
27. Mouhat S, Visan V, Ananthakrishnan S, Wulff H, Andreotti N, Grissmer S, Darbon H, De Waard M, Sabatier JM. K+ channel types targeted by synthetic OSK1, a toxin from *Orthochirus scrobiculosus* scorpion venom. Biochem J. 2005 Jan 1;385(1):95–104.
28. Gurrola GB, Hernández-López RA, Rodríguez de la Vega RC, Varga Z, Batista CVF, Salas-Castillo SP, Panyi G, del Río-Portilla F, Possani LD.Structure, Function, and Chemical Synthesis of *Vaejovis mexicanus* Peptide 24: A Novel Potent Blocker of Kv1.3 Potassium Channels of Human T Lymphocytes. Biochemistry. 2012 May 15;51(19):4049–61.
29. Hashemlou M, Zare Mirakabadi A, Ahmadi M, Hejazi M. Study on anti inflammatory effect of scorpion (*Mesobuthus eupeus*) venom in adjuvant-induced arthritis in rats. Arch Razi Inst. 2009 May 1;64(1):51–6.
30. Tanner MR, Pennington MW, Chamberlain BH, Huq R, Gehrmann EJ, Laragione T, Gulko PS, Beeton C . Targeting KCa1.1 channels with a scorpion venom peptide for the therapy of rat models of rheumatoid arthritis. J Pharmacol Exp Ther. 2018 May;365(2):227–36.
31. Zoccal KF, Bitencourt C da S, Secatto A, Sorgi CA, Bordon K de CF, Sampaio SV, Candiani E, Faccioli LH. *Tityus serrulatus* venom and toxins Ts1, Ts2, and Ts6 induce macrophage activation and production of immune mediators. Toxicon. 2011;57(7–8):1101–8.
32. Petricevich VL, Lebrun I. Immunomodulatory effects of the *Tityus serrulatus* venom on murine macrophage functions *in vitro*. Mediators Inflamm. 2005 Feb 24;2005(1):39–49.
33. Adi-Bessalem S, Hammoudi-Triki D, Laraba-Djebari F. Pathophysiological effects of *Androctonus australis* hector scorpion venom: Tissue damages and inflammatory response. Exp Toxicol Pathol. 2008 Aug;60(4–5):373–80.
34. Morales Duque H. Caracterização de novos peptídeos bloqueadores de canais para K+ isolados da peçonha do escorpião *Tityus* sp. Repos Inst da UNB. 2013 Feb 28 [cited 2019 Sep 8]; Available from: http://repositorio.unb.br/handle/10482/13523.
35. Lowe RM, Farrell PM. A portable device for the electrical extraction of scorpion venom. Toxicon. 2011 Feb;57(2):244–7.
36. Page B, Page M, Noel C. A new fluorometric assay for cytotoxicity measurements in-vitro. Int J Oncol. 1993 Sep 1;3(3):473–6.
37. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 2000 Sep;267(17):5421–6.
38. Solomon DH, Bitton A, Katz JN, Radner H, Brown EM, Fraenkel L. Review: treat to target in rheumatoid arthritis: fact, fiction, or hypothesis? Arthritis Rheumatol (Hoboken, NJ). 2014 Apr 1;66(4):775–82.
39. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010 Sep;62(9):2569–81.
40. Lyons AB. Analysing cell division *in vivo* and *in vitro* using flow cytometric measurement of CFSE dye dilution. J Immunol Methods. 2000 Sep 21;243(1–2):147–54.
41. Petrelli A, Van Wijk F. CD8+ T cells in human autoimmune arthritis: The unusual suspects. Nat Rev Rheumatol. 2016 Jul 1;12(7):421–8.
42. Carvalheiro H, Duarte C, Silva-Cardoso S, da Silva JAP, Souto-Carneiro MM. CD8+ T cell profiles in patients with rheumatoid arthritis and their relationship to disease activity. Arthritis Rheumatol. 2015 Feb 1;67(2):363–71.
43. Kindt T, Goldsby R, Osborne B. Inmunología de los trasplantes. McGraw-Hill Interamericana, editors. Inmunologia de Kuby. Bogotá: McGraw-Hill; 2007. p. 424–46.
44. Lam J, Wulff H. The lymphocyte potassium channels Kv1.3 and KCa3.1 as targets for immunosuppression. Drug Dev Res. 2011 Nov;72(7):573–84.
45. Varga Z, Gurrola-Briones G, Papp F, Rodriguez De La Vega RC, Pedraza-Alva G, Tajhya RB, Gaspar R, Cardenas L, Rosenstein Y, Beeton C, Possani LD, Panyi G. Vm24, a natural immunosuppressive peptide, potently and selectively blocks Kv1.3 potassium channels of human T cells. Mol Pharmacol. 2012 Sep 1;82(3):372–82.
46. Wulff H, Calabresi PA, Allie R, Yun S, Pennington M, Beeton C, Chandy KG. The voltage-gated Kv1.3 K+ channel in effector memory T cells as new target for MS. J Clin Invest. 2003 Jun;111(11):1703–13.
47. Hu L, Pennington M, Jiang Q, Whartenby KA, Calabresi PA. Characterization of the functional properties of the voltage-gated potassium channel Kv1.3 in human CD4+ T lymphocytes. J Immunol. 2007 Oct 1;179(7):4563–70.
48. Arenas CJ. Identificação e caracterização de peptídeos moduladores de canais para sódio presentes na peçonha do escorpião *tityus* sp. pertencente ao grupo forcipula. Tese (Doutorado em Biologia Molecular)—Universidade de Brasília. Universidade de Brasília; 2013.
49. Barona J, Batista CVF, Zamudio FZ, Gomez-Lagunas F, Wanke E, Otero R, Possani LD. Proteomic analysis of the venom and characterization of toxins specific for Na+- and K+-channels from the Colombian scorpion *Tityus pachyurus*. Biochim Biophys Acta. 2006;1764(1):76–84.
50. Guerrero-Vargas JA, Mourão CBF, Quintero-Hernández V, Possani LD, Schwartz EF. Identification and phylogenetic analysis of *Tityus pachyurus* and *Tityus obscurus* novel putative Na +-channel scorpion toxins. PLoS One. 2012 Feb;7(2).
51. Guerrero-Vargas JA, da Silva Libério M, Souza Castro M. Aplicación biotecnológica de la toxinología ; la importancia del veneno de escorpiones y la secre ión cutánea de Anuros. Asociación Colombiana para el avance de la ciencia, editors. Colombia, Innovación Y Ciencia. Colombia, 2008 p. 16-25.
52. Wang X, Li G, Guo J, Zhang Z, Zhang S, Zhu Y, Cheng J, Yu L, Ji Y, Tao J.. Kv1.3 Channel as a Key Therapeutic Target for Neuroinflammatory Diseases: State of the Art and Beyond. Front Neurosci. 2020 Jan 14;13:1393.
53. Zhao Y, Huang J, Yuan X, Peng B, Liu W, Han S, He X.. Toxins targeting the Kv1.3 Channel: Potential immunomodulators for autoimmune diseases. Toxins (Basel). 2015 May 19;7(5):1749–64.
54. Rashid MH, Huq R, Tanner MR, Chhabra S, Khoo KK, Estrada R, Dhawan V, Chauhan S, Pennington MW, Beeton C, Kuyucak S, Norton RS. A potent and Kv1.3-selective analogue of the scorpion toxin HsTX1 as a potential therapeutic for autoimmune diseases. Sci Rep. 2014 Mar 28;4:4509.
55. Corzo G, Espino-Solis GP. Selected scorpion toxin exposures induce cytokine release in human peripheral blood mononuclear cells. Toxicon. 2017 Mar 1;127:56–62.
56. Lebrun B, Romi-Lebrun R, Martin-Eauclaire MF, Yasuda A, Ishiguro M, Oyama Y, Pongs O, Nakajima T. A four-disulphide-bridged toxin, with high

affinity towards voltage-gated K+ channels, isolated from *Heterometrus spinnifer* (Scorpionidae) venom. Biochem J. 1997 Nov 15;328(1):321–7.

57. Veytia-Bucheli JI, Jiménez-Vargas JM, Melchy-Pérez EI, Sandoval-Hernández MA, Possani LD, Rosenstein Y. K v 1.3 channel blockade with the Vm24 scorpion toxin attenuates the CD4 + effector memory T cell response to TCR stimulation. Cell Commun Signal. 2018 Aug 14;16(1):45.
58. Wang X, Dong L, Liang Y, Ni H, Tang J, Xu C, Zhou Y, Su Y, Wang J, Chen D, Mao C. Performance evaluation of FlowCytomix assays to quantify cytokines in patients with rheumatoid arthritis. Int J Clin Exp Med. 2015 Sep 15;8(9):16158–66.
59. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol. 2007 Jun;7(6):429–42.